

## An enhancer element located downstream of the major glutamate dehydrogenase gene of *Bacillus subtilis*

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**ABSTRACT** The *rocG* gene of *Bacillus subtilis*, encoding a catabolic glutamate dehydrogenase, is transcribed by SigL ( $\sigma^{54}$ )-containing RNA polymerase and requires for its expression RocR, a member of the NtrC/NifA family of proteins that bind to enhancer-like elements, called upstream activating sequences (UAS). Unlike the case for other  $\sigma^{54}$ -dependent genes, *rocG* has no UAS; instead, its expression depends on a sequence located 1.5 kilobases downstream of the *rocG* promoter, beyond the end of the *rocG* coding region. The same sequence also serves as the UAS for the downstream *rocABC* operon and can activate *rocG* if moved upstream of its promoter. Furthermore, the activating sequence can be moved as far as 15 kilobases downstream of the *rocG* promoter and still retain partial activity.

Enhancer elements and enhancer-binding proteins are widespread components of transcriptional regulation systems in eukaryotes (1). Enhancer-like elements also have been described in bacteria. In most cases, they are the upstream activating sequences (UAS) required for expression of genes transcribed by RNA polymerases containing  $\sigma^{54}$  (RpoN)-like subunits (2–5). To initiate transcription,  $\sigma^{54}$ -containing RNA polymerases must interact with additional proteins, members of the NtrC/NifA family, that bind to UAS usually located 100–200 bp upstream of the regulated genes (2–5). It has been shown that UAS can be moved 1–2 kilobases (kb) further upstream from the regulated genes without losing their ability to interact with UAS-binding proteins and to activate expression of the regulated genes (6, 7). A UAS placed downstream of the promoter or even on a separate, catenated, plasmid DNA retains its functional activity in an *in vitro* assay (8, 9), but no functional bacterial enhancer-like element has yet been found to have a natural location far downstream of the promoter it regulates, despite the fact that this is a common feature of eukaryotic enhancers (1).

The *Bacillus subtilis* sigL gene encodes a homolog of the  $\sigma^{54}$  subunit of RNA polymerase (10), and three *B. subtilis* UAS-binding proteins, LevR, RocR, and BkdR, have been characterized (11–15). RocR controls two operons, *rocABC* and *rocDEF*, involved in arginine degradation to glutamate (Fig. 1). Both operons have SigL-dependent promoters and are induced by arginine, ornithine, or proline (13, 14, 16). In the present work, we show that the *B. subtilis* *rocG* gene, encoding a major catabolic glutamate dehydrogenase (GluDH) (17), is a member of the RocR regulon. RocG activity (glutamate + NAD  $\rightarrow$  2-ketoglutarate + NH<sub>3</sub> + NADH) can be viewed as the final step in the use of arginine, ornithine, and proline as carbon or nitrogen sources (Fig. 1). Two unique features of *rocG* expression are the location of the apparent RocR binding site downstream of the *rocG* coding region and the ability of this site to act at distances as far as 15 kb from the *rocG* promoter.

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## MATERIALS AND METHODS

**Bacterial Strains and Culture Media.** *B. subtilis* strains used in this study are derivatives of wild-type strain SMY and were constructed by chromosomal DNA or plasmid transformation as described (17). Cells were grown at 37°C in TSS minimal medium (18) with 0.5% glucose or 0.4% succinate as the carbon source and 0.2% of other carbon or nitrogen sources. Other microbiological procedures were as described (17).

**Plasmid and Strain Constructions.** General methods for DNA manipulations and transformation were as described (17). The genetic and restriction maps of the *rocG* locus and some of the plasmids used in this study are presented in Fig. 2. Plasmids were constructed as described below or by deleting or subcloning fragments of the *rocG* region (17, 19).

**Construction of *rocG-lacZ* fusions.** The 1.22-kb *PstI-EcoRI* fragment (Fig. 2) containing the 5'-part of *rocG* and 692 bp upstream of the *rocG* initiation codon was excised from pBB907 (ref. 17; Fig. 2B) with *Bam*HI and *Eco*RI and was cloned in pJPM82 (20). The resulting plasmid, pBB914, contained the *rocG* fragment fused to the promoterless *Escherichia coli* *lacZ* gene in the proper orientation. pBB923 (Fig. 2A) was created from pBB914 by deleting the 3'-terminal 0.16-kb *SacII-EcoRI* fragment of the *rocG* insert. To construct pBB986 and pBB993, the 0.37-kb *Bst*YI-*Bcl*I fragment containing the 3'-end of *rocG* and additional 106 bp of downstream DNA (Fig. 2) was cloned in two orientations in the *Bam*HI site of pBB923 upstream of the *PstI-SacII* insert. *rocG-lacZ* fusions were integrated into the chromosome (Fig. 2A) either at the *amyE* locus by a double-crossover recombination event as described (17) or at the *rocG* locus by a single-crossover recombination event. The location of fusions at the *rocG* locus was verified in genetic crosses with linked markers.

**Construction of a *rocG* null mutant.** A deletion-insertion mutation within the *rocG* gene (pBB956; Fig. 2B) was made in a manner similar to that described for pBB918 (17) by replacing the 0.85-kb *EcoRI-Bcl*I fragment of pBB907 (Fig. 2B) with the 1.6-kb *EcoRI-Bam*HI *ble* cassette, excised from pJPM136 (17). Strain BB1541 containing the  $\Delta(\text{rocG-DAS})::\text{ble}$  allele was constructed as described for strain BB1284 ( $\Delta\text{rocG}::\text{ble}$ ) (17).

Plasmid pBB921 (Fig. 2B) was constructed by cloning the 2.1-kb *PstI-Bcl*I fragment cut from pBB907 (Fig. 2B) with *Bam*HI and *Bcl*I in the *Bam*HI site of pJPM82 (20) (the direction of *rocG* transcription in this plasmid coincides with that of the *lacZ* gene). pBB967 (Fig. 2B) was made in a similar way by cloning a 2.0-kb *Bam*HI-*Bam*HI PCR product that contained the entire *rocG* gene and 9 bp of the downstream region.

Abbreviations: UAS, upstream activating sequence; GluDH, glutamate dehydrogenase; DAS, downstream activating sequence; kb, kilobase.

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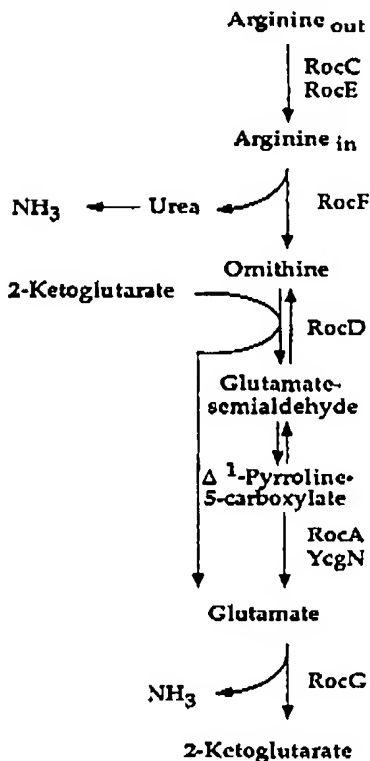


FIG. 1. The pathway for arginine and ornithine use. The following enzymes are shown on this figure by the names of their corresponding genes: RocC and RocE, putative arginino/ornithine permeases; RocF, arginase; RocD, ornithine aminotransferase; RocA and YcgN,  $\Delta^1$ -pyrroline-5-carboxylate dehydrogenases; RocG, glutamate dehydrogenase. Proline is converted to glutamate via  $\Delta^1$ -pyrroline-5-carboxylate. The proline degradation pathway shares RocA/YcgN- and RocG-dependent steps with the roc-pathway.

Plasmid pBB1023, lacking the 106 bp of the downstream activating sequence (DAS)/UAS region, was constructed from pBB907 (17) by replacing the 2.1-kb *Pst*I-*Bcl*I fragment (as in pBB921) with the 2.0-kb *Pst*I-*Bam*HI fragment of pBB967 (Fig. 2B). Strain BB1664 ( $\Delta$ DAS/UAS) has the deletion mutation of pBB1023 in the chromosomal *rocG rocA* locus. It was isolated as a spontaneous derivative of a strain carrying an integrated pBB1023 at the *rocG rocA* locus in which the plasmid (and its neomycin-resistance gene) had been eliminated by recombination. The replacement of the chromosomal *rocG rocA* locus by its  $\Delta$ DAS/UAS allele was confirmed by comparing sizes of the PCR fragments from the wild-type and mutant chromosomal loci.

A *rocD::aphA3::spe* strain was constructed from the *rocD::aphA3* mutant (14) by inactivating the *aphA3* marker by a double-crossover recombination event using pBB838. The latter plasmid was constructed by cloning the 1.1-kb blunt-ended *spe*-cassette, cut from pJL73 (21) with *Bam*HI and *Eco*RI, into the *Blp*I site of the *aphA3* insert of pDG782 (22). The direction of *spe* transcription in pBB838 coincides with that of the *aphA3* gene.

**Enzyme Assays.**  $\beta$ -galactosidase activity was determined as described and was expressed in Miller units (17). GlutDH activity was assayed and expressed in units as described (17).

## RESULTS AND DISCUSSION

**Identification of the *rocG* Promoter.** The results of genetic analysis placed the *rocG* promoter between the *Cla*I site and

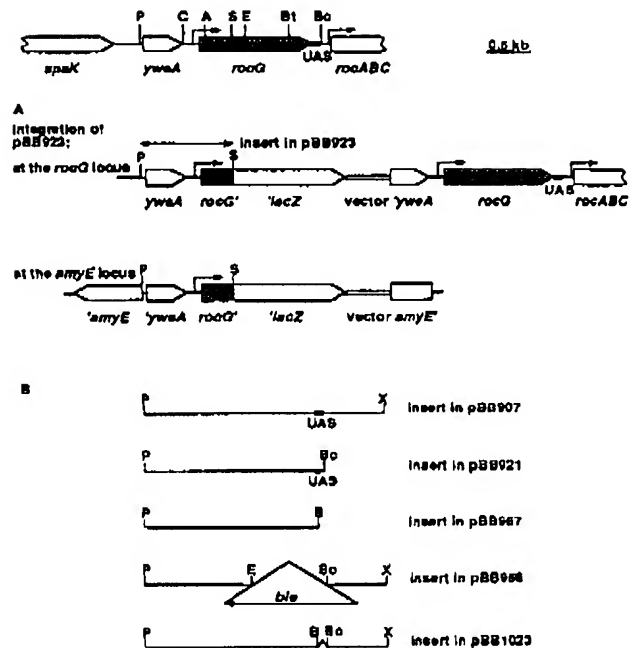
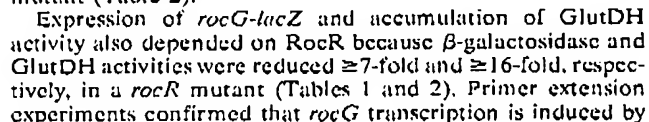


FIG. 2. The genetic map of the *rocG* region (19) and plasmids carrying different parts of this region. The structure of the chromosome resulting from integration of pBB923 at the *rocG* or *amyE* locus is shown. For pBB956, pBB921, and pBB967, such integration events are shown in Fig. 4 (see strains BB1541, 1566, and 1568, respectively). Plasmids pBB907, 956, and 1023 are derivatives of pBB544 (20), and pBB921, pBB923, and pBB967 are derivatives of pJPM82 (20). Plasmid pBB907 was described previously (17). Restriction sites are abbreviated as follows: A, *Acc*I; B, *Bam*HI; Bc, *Bcl*I; Bt, *Bst*YI; C, *Cla*I; E, *Eco*RI; P, *Pst*I; S, *Sac*II; X, *Xho*I. The *Xho*I and *Bam*HI sites were constructed by PCR. The locations of the *rocG* and *rocA* promoter regions are indicated by right-angle arrows. The *lacZ* gene and the vector part of the plasmids are not to the scale.

the *rocG* initiation codon located upstream of the *Acc*I site (Fig. 2). Integration into the chromosome of a plasmid, carrying an internal *Acc*I-*Eco*RI fragment of *rocG* (Fig. 2), disrupted the *rocG* gene and caused inability to use arginine, ornithine, or proline as sole carbon source, characteristic of a *rocG* null mutant (17). In contrast, a strain with an integrated plasmid, carrying the *Cla*I-*Eco*RI fragment (Fig. 2), retained the wild-type growth phenotype and thus was able to express the *rocG* gene.

A primer extension experiment identified two closely spaced, apparent transcription start points for *rocG* at positions G(-87) and G(-85) with respect to the *rocG* initiation codon (Fig. 3).  $\sim 90$  bp downstream of the *Cla*I site, a putative SigL-dependent promoter had been recognized upstream of the *rocG* gene (ref. 19; in ref. 19, the *rocG* gene is referred to as *ipa-75d*) (Fig. 3). Our primer extension results are consistent with use of this promoter for *rocG* expression.

***rocG* Expression Is Repressed by Glucose, Is Induced by Ornithine and Arginine, and Depends on SigL and RocR.** Several *rocG-lacZ* fusions containing the *rocG* promoter region but different *rocG/lacZ* junction points were constructed [pBB923 (Fig. 2A); data not shown] and integrated at the *rocG* locus of the chromosome, a recombination event that provided the normal chromosomal arrangement of DNA sequences upstream of the *rocG* part of the fusion. In glucose minimal medium, these fusions were not active (data not shown), but their activity could be detected in the absence of glucose when succinate served as carbon source and ornithine, arginine, or



Both the longer and the shorter constructs [as in pBB921 and pBB967, respectively (Fig. 2B)] were able to complement the growth defect caused by the *rocG* null mutation when integrated at the *rocG* locus (Fig. 4, strains BB1562 and BB1564), although, in BB1564, complementation was much less effective. In strain BB1564, the 3'-end of the intact *rocG* gene is separated from the DAS by  $\approx 13.5$  kb. Interestingly, this result implies either that the DAS can act at a distance of  $\approx 15$

Table 2. RocG-GlutDH activity

Strain (genotype)	Carbon and nitrogen sources	RocG activity
BB1268 (wild-type)	Succinate + glutamate	<40
BB1268 (wild-type)	Succinate + glutamate + ornithine	649
BB1552 ( <i>sigL::aphA3</i> )	Succinate + glutamate + ornithine	<40
BB1553 ( <i>rocRΔ3::aphA3</i> )	Succinate + glutamate + ornithine	<40
BB1542 [ <i>rocR</i> (T120I)]	Succinate + glutamate	763
BB1542 [ <i>rocR</i> (T120I)]	Succinate + glutamate + ornithine	829
BB1680 ( <i>ahrC::aphA3</i> )	Succinate + glutamate + ornithine	<40

The null mutations in *sigL*, *rocR*, *rocD*, and *ahrC* and the constitutive *rocR*(T120I) mutation were as described (10, 13, 14). All strains carried the  $\Delta$ *gudB::tet* mutation to avoid any contribution from *GudB*-GlutDH(17).

kb from the *rocG* promoter (Fig. 4, strain BB1564) or that sequences upstream of *rocG*, which are absent at the *amyE* locus but present at the *rocG* locus, can compensate for the lack of the DAS.

To test whether sequences upstream of *rocG* can substitute for the DAS, we created a  $\Delta$ (*rocG*-DAS)::*hle* deletion-insertion chromosomal mutant (Fig. 4, strain BB1541) lacking the 3'-coding region of *rocG* and 106 bp of the downstream region [as in pBB956 (Fig. 2B)]. As was the case for the previously described *rocG::hle* mutation, the new, extended deletion was complemented both at the *amyE* and *rocG* loci by a *rocG* construct containing the DAS (Fig. 4, strain BB1566; data not shown). The  $\Delta$ (*rocG*-DAS)::*hle* deletion was not complemented by a construct containing the entire coding sequence but lacking the DAS, even when the DAS-less construct was integrated in cis at the *rocG* locus (Fig. 4, strain BB1568; data not shown). Thus, the sequences upstream of *rocG* cannot substitute for the lack of the DAS (Fig. 4, strain BB1568). To finally prove the necessity of the DAS for *rocG*

expression, we made a chromosomal deletion of the 106-bp DAS region [as in pBB1023 (Fig. 2B)]. Strain BB1664 ( $\Delta$ DAS/UAS) containing such a deletion had no detectable GlutDH activity but could be complemented by a DAS-associated *rocG* gene [as in pBB921 (Fig. 2B)] located at the *amyE* locus (Table 3).

We conclude that high glutamate dehydrogenase activity was detected in strains with integrated *rocG* plasmids when the DAS was immediately downstream of *rocG*. Lower, but still significant, activity was seen when the DAS was separated from the *rocG* promoter by 15 kb of intervening sequence; no activity was detected in the absence of the DAS (Fig. 4). An intermediate GlutDH activity (~150 units) was detected when the DAS was placed 6.3 kb downstream of the *rocG* promoter (a plasmid of 4.8 kb containing the 0.45-kb 3'-fragment of *rocG* without the DAS was integrated into the chromosome of a wild-type strain). In other words, the presence of the DAS in its natural position immediately downstream of *rocG* (1.5 kb downstream of the promoter) or at a distance as far as 13.5 kb

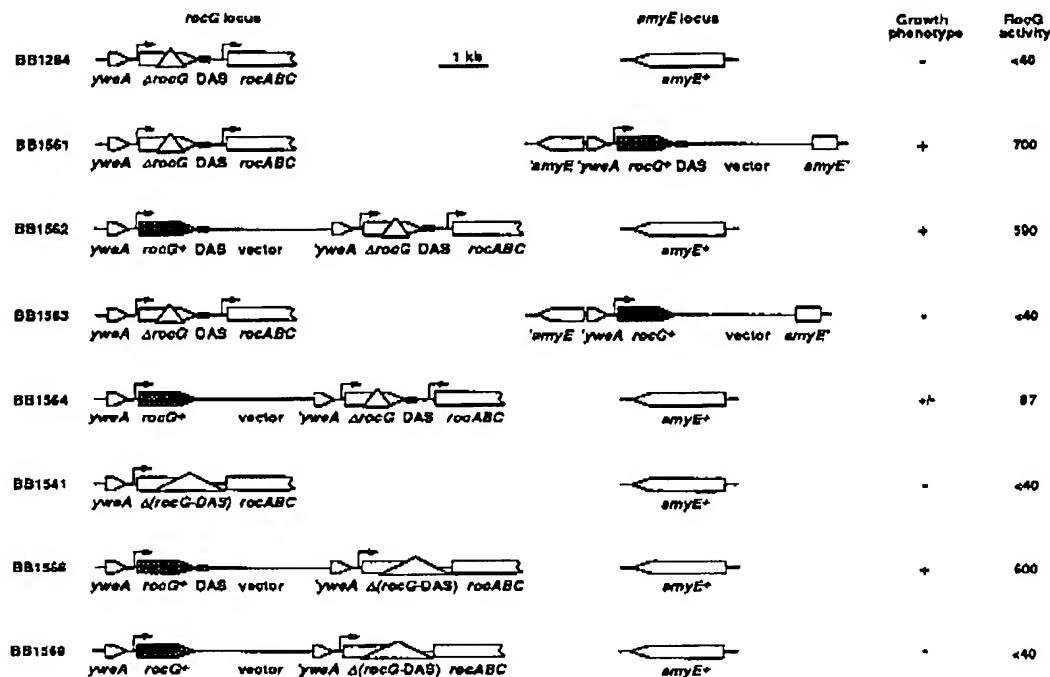


FIG. 4. Requirement of DAS for complementation of *rocG* mutations. Plasmids pBB921 or pBB967 (Fig. 2B) were integrated in the chromosome of two different *rocG* deletion mutants (strains BB1284 and BB1541) either at the *rocG* locus or at the ectopic *amyE* locus. The full-length *rocG* allele and the DAS are presented as filled boxes. The vector part of the plasmids (10.5 kb) and the DAS (61 bp) are not to scale. The locations of the *rocG* and *rocA* promoter regions are indicated by right-angle arrows. Growth phenotype was scored as ability to use ornithine or proline as sole carbon and nitrogen source. GlutDH activity was determined in succinate-glutamate-ornithine medium.

Table 3. RocG-GlutDH activity in the absence of the DAS

Strain (genotype)	RocG activity
BB1664 ( $\Delta$ DAS/UAS)	<40
BB1667 [ $\Delta$ DAS/UAS <i>amyE::rocG</i> (DAS/UAS <sup>+</sup> )]	683
BB1668 [ $\Delta$ DAS/UAS <i>amyE::rocG</i> (DAS/UAS <sup>+</sup> )]	<40
BB1665 ( $\Delta$ DAS/UAS <i>rocD::uphA3::spe</i> )	65
BB1666 ( $\Delta$ DAS/UAS <i>rocD::uphA3::spe rocR<math>\Delta</math>3::uphA3</i> )	<40

Strain BB1664 containing the 106-bp DAS/UAS deletion and its derivatives were grown in succinate-glutamate-ornithine medium. The null mutations in *rocD* and *rocR* were described, respectively, in *Material and Methods* and in ref. 13. All strains carried the  $\Delta$ *gudB::tet* mutation to avoid any contribution from GudB-GlutDH (17).

farther downstream of *rocG* is a prerequisite for *rocG* expression.

In the *rocG-lacZ* fusion used in Table 1, the DAS is separated from the *rocG* promoter by 13 kb of plasmid and *rocG* sequences (Fig. 2A). This separation presumably explains the low activity of this fusion. The relatively small effect of the *rocR*(T1201) mutation on *rocG-lacZ* expression (compare Tables 1 and 2) also could be attributable to separation of the *rocG* promoter from the DAS.

The DAS Overlaps the *rocABC* UAS and Behaves as an Enhancer. The 106-bp sequence immediately downstream of *rocG* contains the previously described 61-bp UAS (but not the promoter) of the *rocABC* operon (Fig. 2). This UAS is a double dyad symmetry element and is a putative binding site for RocR (13, 14, 16). [The phenotypes of strains BB1541 (Fig. 4), BB1664 (Table 3), and their derivatives carrying the *rocG* (DAS/UAS<sup>+</sup>) gene at the *amyE* locus indicate that expression of the *rocABC* operon, which should be abolished as a result of the UAS deletions, is not required for use of arginine, ornithine, or proline and does not affect GlutDH activity.] To show more directly that this sequence is important for *rocG* activity and can act as a position- and orientation-independent enhancer element for the *rocG* gene, we relocated the DAS/UAS upstream of a *rocG-lacZ* fusion and integrated the construct at the *amyE* locus. The presence of the DAS/UAS in the upstream position [as in pBB986 (Fig. 5)] greatly stimulated expression of  $\beta$ -galactosidase (compare Tables 1 and 4) [Positioning of the *rocABC* UAS upstream of another SigL-dependent promoter, *levD*, also activated its expression (14)].

Expression of the fusion with the DAS/UAS in the upstream position was still subject to glucose repression (data not shown) and depended for its induction on SigL and RocR and arginine, ornithine, or proline (Table 4; data not shown). Note that, with this construct, the constitutive *rocR* mutation allowed high ornithine-independent expression of the *rocG-lacZ* fusion, unlike the case in which the DAS/UAS was separated from the *rocG* promoter by an abnormally great distance (compare Tables 1 and 4). Ability of the DAS/UAS to activate *rocG* in the upstream position was independent of its orientation or its precise position with respect to the *rocG* promoter (Fig. 5). These features were previously demonstrated for UAS of other members of the NtrC/NifA family (6, 7, 30–34). The ability of the DAS/UAS to activate *rocG* from either an upstream or a downstream position makes it very unlikely that stability of *rocG* mRNA is involved in the activation phenomenon.

The DAS Requirement for *rocG* Expression Can Be Bypassed. In otherwise wild-type cells, *rocG* expression requires the DAS positioned either at its natural location downstream of the gene or relocated upstream or farther downstream of *rocG*. However, in a *rocD* mutant, defective in ornithine aminotransferase (Fig. 1), this requirement for the DAS was partially alleviated. In such a mutant, a *rocG-lacZ* fusion lacking the DAS and integrated at the *amyE* locus was more active in cells grown in the presence of ornithine (Table 1).

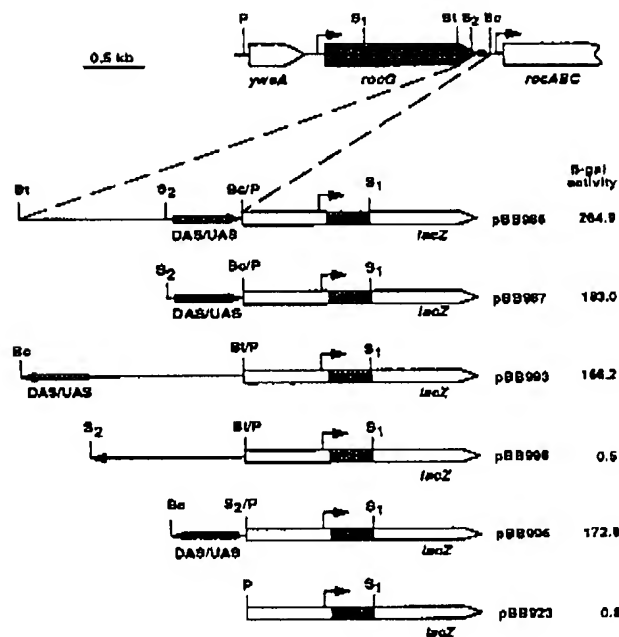


FIG. 5. The *rocG* DAS behaves as a position- and orientation-independent enhancer. Derivatives of strain SMY carrying the corresponding *rocG-lacZ* fusions at the ectopic *amyE* locus were grown in succinate-glutamate-ornithine medium, and  $\beta$ -galactosidase activity was determined. The *lacZ* gene and the enlarged fragments of the DAS/UAS region are not to the scale. The arrows show orientation of the DAS/UAS region. The locations of the *rocG* and *rocA* promoter regions are indicated by right-angle arrows. The restriction sites are abbreviated as follows: Bc, *Bcl*I; Bt, *Bst*YI; P, *Pst*I; S, *Sac*II.

Moreover, in the absence of RocD, a full-length *rocG* gene lacking the DAS [*rocD* mutant versions of strains BB1563 or BB1664 (Fig. 4 and Table 3)] could provide measurable GlutDH activity (65–120 units). It was shown previously that RocR protein is activated by ornithine and that RocR-dependent expression of the *rocDEF* operon in ornithine-containing medium is higher in a *rocD* mutant than in a wild-type strain (14). It seems likely that a defect in ornithine aminotransferase, catalyzing interconversion of ornithine and  $\Delta^1$ -pyrroline-5-carboxylic acid (Fig. 1), leads to intracellular accumulation of ornithine in ornithine-containing medium. Under these conditions, RocR may adopt a more active conformation that allows the protein to bind to low affinity sites or to interact with SigL without binding to DNA. The DAS-independent, ornithine-stimulated expression of *rocG* still depended on RocR (Table 3). Conditions that allow other proteins of the NtrC/NifA family to activate transcription in

Table 4. Expression of a *rocG-lacZ* fusion containing the DAS/UAS in the upstream position

Strain (genotype)	$\beta$ -galactosidase activity in different media	
	Succinate Glutamate	Succinate Glutamate Ornithine
BB1501 (wild-type)	2.1	264.9
BB1503 ( <i>sigL::uphA3</i> )	1.2	1.2
BB1502 ( <i>rocR<math>\Delta</math>3::uphA3</i> )	2.0	1.4
BB1522 [ <i>rocR</i> (T1201)]	122.6	225.3

Strain BB1501 and its derivatives carry a *rocG-lacZ* fusion [as in pBB986, (Fig. 5)] at the *amyE* locus.

the absence of their respective UAS have been extensively described (7, 35–37).

**Conclusions and Implications.** The presence of a functional enhancer element downstream of a  $\sigma^{54}$ -dependent gene is unique to the *B. subtilis rocG* gene. The single NtrC binding site upstream of the *E. coli glnL* gene and immediately downstream of the *gluA* gene does not contribute to the NtrC-dependent activation of the latter gene *in vivo* (7), although it can activate *gluA* if placed upstream of the *gluAp2* promoter (38). In most other cases, the corresponding elements are positioned upstream of the regulated promoters (2–5). In several cases of divergently transcribed  $\sigma^{54}$ -dependent genes, enhancer-like elements are found just downstream of the promoters in the 5' untranslated region or within the N-terminal coding regions of the regulated genes (39, 40). In another case, no UAS could be identified upstream of the *argT* promoter of *Salmonella typhimurium*, but neither the presence nor the location of any AS could be ascertained (41). An attempt to move the *nifH* UAS downstream of the *nifH* translation start did not result in an active construct (42). The apparent ability of the RocR-DAS complex to influence promoter activity from as far away as 15 kb downstream is an unusual example of enhancer action-at-a-distance.

Another unusual feature of the *rocG* enhancer element is that it is shared by the *rocG* gene and the downstream *rocABC* operon. We imagine that RocR bound to the DAS/UAS interacts independently with molecules of SigL-containing RNA polymerase at the *rocG* promoter, located  $\approx 1,450$  bp upstream, and the *rocABC* promoter, located  $\approx 100$  bp downstream. Both interactions likely require DNA looping (43, 44), although the strengths and geometries of these interactions may be different. In a remarkable case of sequence economy, the DAS/UAS appears also to be the transcription terminator for *rocG*.

*rocG* is not completely coregulated with the other *roc* genes. In contrast to the *rocG* gene, which is repressed in glucose-containing media by CcpA, the *rocABC* and *rocDEF* operons are highly expressed in minimal glucose medium (13, 16) and are not significantly regulated by CcpA (data not shown). This differential regulation of the genes of the *roc* regulon could reflect different functional roles of the corresponding products. Although RocF and RocD liberate nitrogen groups from arginine and ornithine, respectively, RocG has a dual role, providing both ammonia as a nitrogen source and 2-ketoglutarate as a carbon source (Fig. 1), and may therefore be subject to more complex regulation. Moreover, full RocG activation could lead to depletion of the pool of glutamate, a major cellular anion (45); cells may only allow glutamate degradation in the absence of rapidly metabolizable carbon sources. It will be interesting to find out whether the downstream position of the *rocG* DAS is a prerequisite for differential regulation of the *rocG* and other *roc* genes.

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